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- 1) Winkles J et al., Enhanced-latex-agglutination assay for C-reactive protein in serum, with use of a centrifugal analyzer. Clinical chemistry, (1987 May) 33 (5) 685-9.
- 2) Kitahashi S. et al.; Diagnosis of infections in newborns using a new particle-mediated immunoassay for serum C-reactive protein. Journal of Automatic Chemistry. (1998) 20/6 (195-198).
- 3) Ueno T. et al., Liposome turbidimetric assay (LTA). Advanced Drug Delivery Reviews, (1997) 24/2-3 (293-299).

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Article title	Liposome turbidimetric assay (LTA)
Article identifier	0169409X97001872
Authors	Ueno_T Tanaka_S Umeda_M
Journal title	Advanced Drug Delivery Reviews
ISSN	0169-409X
Publisher	Elsevier Netherlands
Year of publication	1997
Volume	24
Issue	2-3
Supplement	0
Page range	293-299
Number of pages	7
User name	Adonis
Cost centre	
PCC	\$20.00
Date and time	Wednesday, March 03, 2004 10:29:55 AM

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Liposome turbidimetric assay (LTA)

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Abstract

We developed a rapid and sensitive liposome turbidimetric assay (LTA) for determining C-reactive protein (CRP) in serum. The assay system was based on the increase of the turbidity induced by the reaction of anti-CRP antibodies-bearing liposomes with CRP antigen, and the assay procedure was fully automated on a Hitachi 717 analyzer. The method had an analytical range of 2–120 mg/l. The results of within-run and between-run precision studies indicated that this system is accurate and gives reproducible data ($<3.0\%$ and $<6.0\%$, respectively). The assay detection limit was less than 1 mg/l. There was no interference from bilirubin, hemoglobin, intrafat, rheumatoid factor, or high- γ -globulin. Furthermore, our results showed good agreement with those obtained using the Bebring nephelometer analyzer ($n = 100$, $r = 0.98$). The LTA using a Hitachi 717 automated analyzer was a convenient method, and represented an interesting alternative to other immunoassays for measuring CRP in serum.

Keywords: CRP antigen; Turbidity; Monoclonal antibody; Automated analyzer; Agglutination; Liposomes bearing antibodies

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1. Introduction

C-Reactive protein (CRP), one of the acute phase reactants, is associated with processes of inflammation, tissue destruction and malignant neoplasia [1–3]. Serum level of CRP is less than 4 mg/l in healthy adults and increases as much as 100-fold in response to inflammatory stimuli [4]. Measurement of CRP in serum is therefore valuable for the diagnoses of various diseases.

Various immunological methods such as semiquantitative capillary tube immunoprecipitation and passive latex agglutination have been developed for the measurement of CRP [5,6]. However, these methods are not available for the quantitative determination. Recently laser nephelometry, latex turbidimetry, and turbidimetric immunoassay (TIA) have been used for the quantitative determination of CRP [7]. In particular, TIA is widely used in many clinical laboratories since it is simple and fast, and can be easily applied to automated analyzers. However, some problems are encountered during practical use of this method, TIA, i.e., the sensitivity of TIA is limited to the detection of over 5 mg/l of CRP, and within-run precision is not enough for the judgment of negative or positive, assuming that values of over 5 mg/l are CRP positive. Although the turbidimetric assay system using a latex carrier has been developed to overcome this disadvantage, it has another problem in that latex particles composed of polystyrene are hydrophobic, which causes nonspecific agglutination and cuvette contamination of automated analyzers.

Taking foregoing the problems into consideration, we attempted to develop liposome turbidimetric assay (LTA) using polyacrylamide-entrapped liposome particles instead of latex because non-specific agglutination and cuvette contamination can be prevented by using liposomes having a hydrophilic surface. We report here a simple, rapid and sensitive LTA for the automated measurement of CRP in serum using a Hitachi 717 automated analyzer.

2. Materials and methods

2.1. Lipids and other chemicals

Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Nippon Oil and Fats Co., Ltd. (Tokyo Japan), and cholesterol (Chol) was obtained from sigma Chemical Co. (St. Louis, MO). These lipids were dissolved in chloroform or chloroform/methanol (2:1), and stored at -20°C under nitrogen gas. *N*-Hydroxysuccinimidyl-3-(2-pyridyldithio)propionate (SPDP) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Acrylamide, *N,N'*-methylene-bis(acrylamide) (BIS), ammonium peroxodisulfate and *N,N,N',N'*-tetramethylethylenediamine (TMED) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2. Haptenized DPPE

3-(2-Dithiopyridyl)propionyl (DTP)-DPPE was prepared from DPPE and SPDP according to the method of Leserman et al. [8] with minor modifications. Briefly, SPDP was treated with DPPE in a chloroform/methanol mixture (3:1) containing 0.1 mol/l triethylamine. After an overnight incubation at room temperature, solvents were removed by evaporation under vacuum and the residue was dissolved in a chloroform/methanol mixture (3:1). Subsequent addition of water resulted in separation into two phases. The lower phase was collected and applied to silica-gel chromatography, and eluted with a chloroform/methanol mixture (5:1). Purity of the preparations was confirmed by thin layer chromatography (chloroform/methanol/water, 65:30:5) which revealed a single spot on silica-gel plates. The obtained lipid was dissolved in chloroform/methanol (5:1), and stored at -20°C under nitrogen gas.

2.3. Purification of CRP (working standards)

Pooled inflammatory human sera containing more than 50 mg/l of CRP were filtered with a $0.45\text{-}\mu\text{m}$

Millipore filter and purified by affinity chromatography with agarose gel coupled to 2-aminoethanol dihydrogenphosphate ligand [9]. CRP prepared here showed a single protein band on electrophoresis using 7.5% SDS-polyacrylamide gel. CRP concentration was determined by single radial immunodiffusion (SRID) (*N*-Immunoring CRP, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). The stock CRP standard was stored at -75°C and a series of working standards was prepared by appropriately diluting the stock with physiological saline before use.

2.4. Modification of two kinds of monoclonal antibodies (IgG fraction)

In order to covalently couple antibodies to liposomes, we selected two monoclonal antibodies (A and B) having high reactivity against CRP and recognizing distinct antigenic determinants. The individual antibody solutions (10 mg/ml) were mixed at a ratio of A/B of 5:1 because the optimal linearity of standard curve was obtained when a 5:1 ratio of the two antibodies was used for coupling.

The mixture of the two monoclonal antibodies was modified with a heterobifunctional cross-linking reagent, SPDP in the following manner: 10 mg/ml of the mixture were incubated in 0.01 mol/l Hepes-buffered saline (pH 7.4) containing 0.9 mmol/l SPDP for 30 min at room temperature. Unreacted SPDP was removed by passage through a Sephadex G-25 column equilibrated with 0.01 mol/l phosphate-buffered saline (pH 6.0), and IgG introduced DTP was reduced with 0.01 mol/l dithiothreitol (DTT) in 0.01 mol/l phosphate-buffered saline (pH 6.0) for 20 min at room temperature. The reaction mixture was passed through a Sephadex G-25 column equilibrated with 0.01 mol/l phosphate-buffered saline (pH 6.0) to remove excess of DTT. The IgG modified with SPDP and DTT was immediately used to prepare antibodies-bearing liposomes.

2.5. Preparation of polyacrylamide-entrapped liposomes

Multilamellar liposomes were prepared from a lipid solution containing DPPC (100 μmol), Chol (100 μmol) and DTP-DPPE (10 μmol). The lipid solution was evaporated to dryness in a rotary

evaporator at 40°C and then further dried for 1 h under vacuum. Acrylamide-entrapped liposomes were obtained by voltexing the lipid film with 800- μm glass beads in 7 ml of 2.5% acrylamide solution. Liposomes having an average diameter of 130 nm were prepared by filtering through 200-nm polycarbonate filter five times, followed by dialysis against 0.01 mol/l phosphate-buffered saline to remove unentrapped acrylamide. After that, ammonium peroxodisulfate and TMED were added to the liposome suspension in order to polymerize acrylamide entrapped in liposomes, and subsequently allowed to stand overnight at room temperature. After the final dialysis against 0.01 mol/l phosphate-buffered saline to remove excess of ammonium peroxodisulfate and TMED, the liposome suspension was adjusted to 0.01 mol/l of phospholipid.

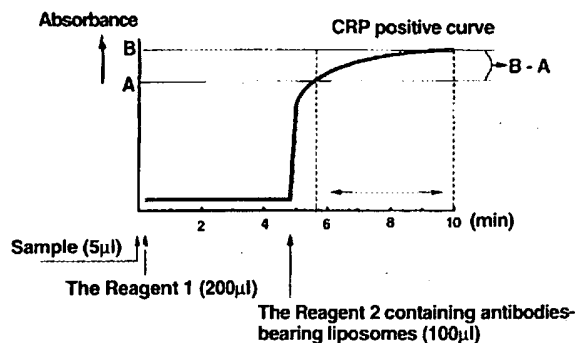
2.6. Preparation of liposomes bearing anti-CRP antibodies

A 5-ml portion of freshly prepared liposome suspension (0.01 mol/l of phospholipid) was added to 5 ml of mouse monoclonal IgG modified with SPDP and DTT at a concentration of 4 mg/ml, and then incubated for 3 days at room temperature with gentle shaking. Unbound IgG was removed by passage through a Sepharose CL4-B column equilibrated with 0.05 mol/l phosphate buffer containing 0.3 mol/l NaCl (pH 6.0). The fractions of liposomes bearing antibodies were collected, and adjusted to a concentration corresponding to 0.0015 mol/l of phospholipid.

2.7. Assay procedure

Sample processing, pipetting steps, and quantifications were performed automatically on a Hitachi 717 analyzer (Hitachi Instruments Engineering Co., Ltd., Ibaraki, Japan).

Five μl of a serum sample and 200 μl of reagent 1 (Tris-HCl-buffered saline, pH 7.5), which was used for stabilization of sample blank, were injected into a reaction cuvette. After a 5-min incubation at 37°C , 100 μl of reagent 2, containing liposomes bearing anti-CRP antibodies in 0.05 mol/l phosphate buffer, containing 0.3 mol/l NaCl (pH 6.0) were added to the cuvette. After another 5 min, CRP concentration was estimated from the difference in absorbance



Scheme 1. Assay procedure. Assay was performed according to the Hitachi 717 instrument settings. Sample blank was stabilized by a 5-min incubation with Reagent 1. After that, CRP concentration was estimated from the difference in absorbance between A and B.

between A and B (Scheme 1) at two wavelengths (main-wavelength: 340 nm, sub-wavelength: 700 nm). Furthermore, a linear calibration curve prepared using one CRP standard serum was used to calculate values of serum samples.

2.8. Principle

Liposome particles covalently coated with two kinds of mouse monoclonal antibodies against human CRP aggregate upon binding with samples containing CRP, thereby forming larger particles which may increase the absorbance. The difference in the absorbance between 340 nm and 700 nm is measured after 5 min and 10 min later. The difference between these two signals is used to establish the standard curve and to calculate values of serum samples as illustrated in Scheme 1. Results are evaluated by the instrument using linear function.

3. Results and discussion

3.1. Effect of affinity on the sensitivity of LTA

We examined the effect of antibody affinity on the sensitivity of LTA. Using two liposome reagents prepared from each of the antibodies having different affinities, a series of purified CRP was measured on a Hitachi 717 automated analyzer. As shown in Fig. 1, when antibody having a high affinity against CRP is used for the LTA, high sensitivity is observed, and

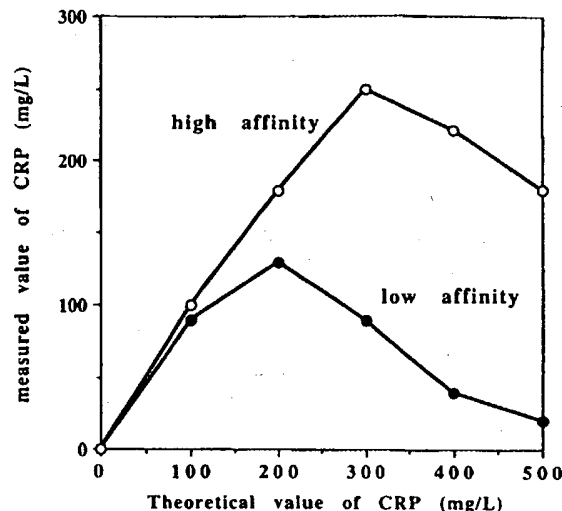


Fig. 1. Effect of affinity on the sensitivity of LTA. A series of known values of CRP was measured on a Hitachi 717 automated analyzer, and the effect of affinity of antibody coupled to liposomes on the sensitivity of LTA was examined.

furthermore the assay system is not susceptible to an antigen excess phenomenon which is an unexpected decrease in turbidity which occurs when the antigen concentration exceeds a certain limit. It is an important that there be no susceptibility to the antigen excess phenomenon for clinical use, because it may result in a false negative outcome. Therefore, we used high affinity mouse monoclonal antibodies to get a high sensitivity and avoid the antigen excess phenomenon.

3.2. Effect of isoelectric point on the sensitivity of LTA

The difference in isoelectric points affects the linearity of the CRP calibration curve. To examine the effect of the isoelectric point on the calibration linearity, two monoclonal antibodies having the same affinity and different isoelectric points, 5.3 and 6.8, were separately coupled to liposomes, and a series of CRP was assayed using the two reagents obtained. As shown in the Fig. 2, in the case of isoelectric point 5.3, the sensitivity of the assay was low in the low level CRP range, and gradually increased with increased CRP concentration, and so calibration resulted in concave curve. On the other hand, in the case of an isoelectric point of 6.8, the sensitivity

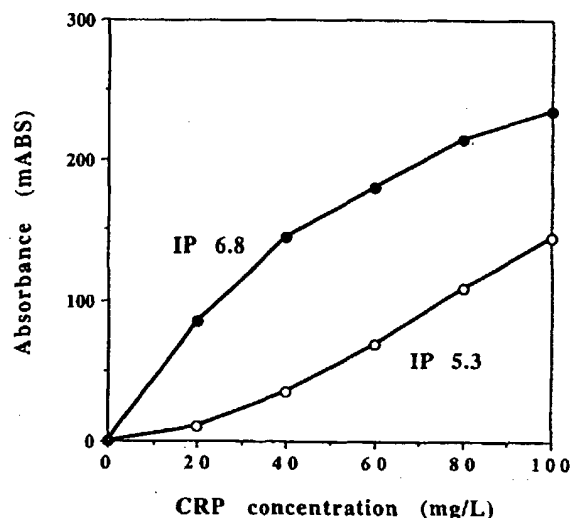


Fig. 2. Effect of isoelectric point on the sensitivity of LTA. To examine the effect of the isoelectric point of antibody on a calibration curve, a series of CRP was assayed using liposome reagents prepared from antibodies having different isoelectric points.

increased from low level CRP, and the calibration curve was convex. When the same experiment was undertaken using other antibodies, the same phenomenon was obtained. The surface of liposomes bearing antibodies having an isoelectric point of 5.3 is more negative than that of liposomes bearing antibodies with an isoelectric point of 6.8 in the reaction buffer adjusted to pH 7.5. Accordingly, the sensitivity in the low level range of CRP may be reduced by repulsion between each liposome, in case of using antibodies having isoelectric point 5.3.

As a result, liposomes bearing two monoclonal antibodies having different isoelectric points were needed in order to get a good linearity in the calibration curve.

3.3. Effect of liposome size on the sensitivity of LTA

To examine the liposome size effect, different sized liposomes were prepared in the range 100–1000 nm, and antibodies were covalently coupled to each of the prepared liposomes. The result of assaying a series of CRP standards using the liposome reagents showed that sensitivity increases with an increase in liposome size in the low level CRP range,

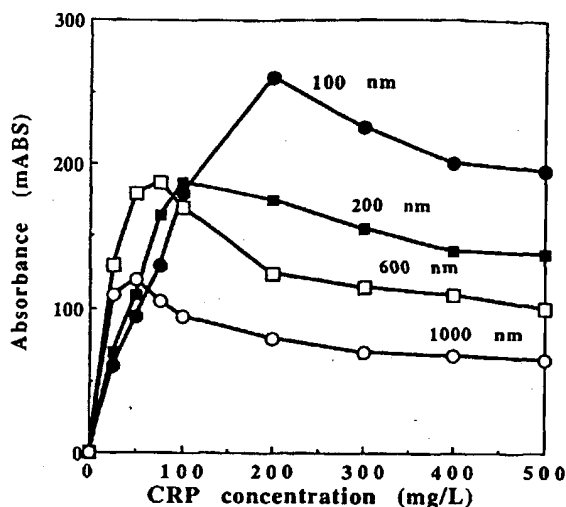


Fig. 3. Effect of liposome size on the sensitivity of LTA. To examine the liposome size effect, liposomes of different sizes were prepared, and a series of CRP was measured with the prepared liposome reagents.

as seen from the data on 100- and 600-nm liposomes (Fig. 3). On the other hand, since liposomes of more than 200 nm were susceptible to the antigen excess phenomenon, considering both the low level CRP range sensitivity and the antigen excess phenomenon, a liposome size of 130 nm was selected.

3.4. Effect of entrapped material on the sensitivity of LTA

Since it is generally known that the refraction index of particles affects the intensity of turbidity, we attempted to entrap water-soluble materials having relatively larger refraction indices than those of water, such as dextran, polyethylene glycol, acrylamide and so on. As shown in Fig. 4, by using acrylamide-entrapped liposomes, the sensitivity reached about twice as high as that of buffer-entrapped liposomes. Although the data are not shown in detail here, when other materials were entrapped in liposomes, roughly the same results were obtained. The reason for entrapping acrylamide in liposomes is to stabilize them by polymerizing the acrylamide. There was no difference in sensitivity between acrylamide and polyacrylamide.

In the following sections, we describe the evaluation of the LTA reagent regarding the measurement

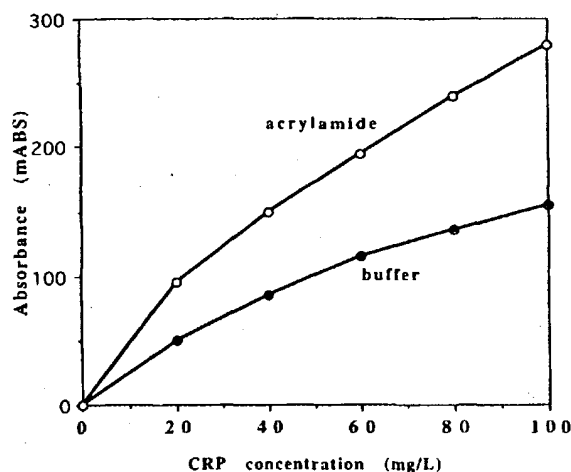


Fig. 4. Effect of entrapped-material on the sensitivity of LTA. The sensitivity of LTA obtained with acrylamide-entrapped liposomes was compared with that of buffer-entrapped liposomes.

of CRP. Unless otherwise stated, polyacrylamide-entrapped liposomes bearing two monoclonal antibodies having different isoelectric points were used for assays.

3.5. Linearity

Fig. 5 shows a representative linear calibration curve obtained with a series of purified CRP. A series of diluted CRP was assayed according to the analytical parameters on a Hitachi 717 automated analyzer, and measurement values were plotted against theoretical values. As shown in Fig. 5, the linear calibration curve was obtained in the range of 2–120 mg/l.

3.6. Antigen excess

The antigen excess phenomenon was examined by assaying a series of diluted CRP. The phenomenon was not observed up to 400 mg/l. The result indicates that the LTA is suitable for diagnostic use.

3.7. Precision

Within-run precision was determined by assaying 10 replicate samples with two different CRP concentrations, and the obtained results were as follows: mean, 5.1 and 20.3; standard deviation (S.D.), 0.2

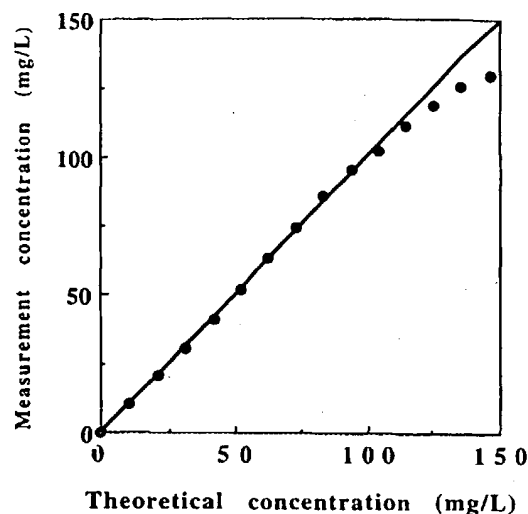


Fig. 5. Linear calibration curve. A series of CRP was assayed according to analytical parameters on a Hitachi 717 automated analyzer, and measurement values were plotted against theoretical values.

and 0.1; coefficients of variation, 3.70 and 0.66, respectively. Although data are not shown here, between-run precision was determined by assaying the same samples in the same manner on 7 consecutive days, and the obtained coefficients of variation were <6.0%. These results indicated that the LTA is accurate and the results are reproducible.

3.8. Detection limit

Physiological saline was measured in 10 replicates, and the S.D. of the measurement values was calculated. When the detection limit was defined as the mean + 3S.D., it was <1 mg/l; this indicates that the LTA is sensitive.

3.9. Interference

We examined the effects of free bilirubin, conjugated bilirubin, hemoglobin, lipemia, rheumatoid factor, and γ -globulin on CRP measurement by using serum samples supplemented with each component. The results showed that bilirubin did not affect the precision of the assay up to 200 mg/l. Similarly, up to 6 g/l of hemoglobin and up to 2700 degrees of lipemia did not affect the precision of the assay.

It is generally known that rheumatoid factor and

γ -globulin affect immunoassays based on the agglutination reaction, because they accelerate non-specific agglutination owing to hydrophobic binding. However, none of them (up to 100 g/l of γ -globulin, and up to 100 IU/ml) affected the precision of the assay in the case of LTA. This may be because the liposome surface is hydrophilic.

3.10. Correlation

Method comparison studies were performed on 100 patient sera with the LTA (y) and the Behring nephelometric latex immunoassay (x). Samples were assayed according to each instrument parameter, and the result showed that there is a good correlation between the two methods ($y = 1.03x - 0.15$, $r = 0.99$).

3.11. Cuvette contamination

We demonstrated the usefulness of the LTA differing from latex reagents. When non-exclusive use automated analyzers such as a Hitachi 717 are used for immunoassays together with biochemical assays, adsorption of immunoreagents, or immunoreactants onto cuvettes is a serious problem. As already stated, the liposome surface is hydrophilic. Therefore, it is anticipated that adsorption caused by hydrophobic binding is avoided. To demonstrate this point, cuvette contamination was checked by measuring the absorbance of the cuvette itself at 340 nm after serum samples were assayed using an LTA reagent, or a latex reagent in the same cuvette, 30 times. The cuvette was automatically washed with distilled water every time. Fig. 6 shows that the run of the LTA reagent does not induce cuvette contamination as compared with that of the latex reagent. Furthermore, although the data are not shown, liposome reagents did not affect measurements of lipid items such as cholesterol, triglyceride and so on.

This assay system (LTA) using the Hitachi 717 automated analyzer is a convenient method, and represents an interesting alternative to other immunoassays for measuring CRP in serum. Furthermore, the above results showed that the LTA is suitable for the determination of CRP from a clinical point of view. We are now continuing our studies using LTA to assay other serum components.

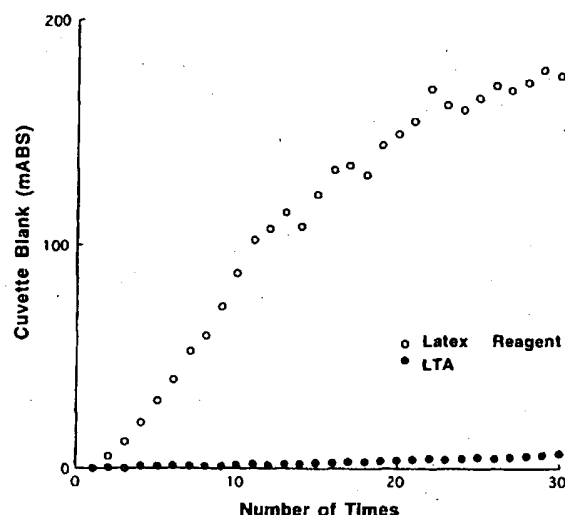


Fig. 6. Cuvette contamination test. Cuvette contamination was checked by measuring the absorbance of the cuvette itself at 340 nm after serum samples were assayed using an LTA reagent, or a latex reagent in the same cuvette 30 times.

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